Synthetic Electric Microbial Biosensors

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14. ABSTRACT
The goal of this research project was for the PI to develop an electrically integrated microbial biosensor using model electroactive microbes through synthetic biology. The PI and his team were very sucessful in this project.

There were four main research goals: 1)to evaluate standard promoter-based sensor inputs and outputs in electroactive microbes 2) To develop electrically detectable signal outputs in electric microbes 3) To develop electrically detectable signal outputs in electric microbes 4) To combine sensor inputs and electrical outputs in electroactive microbes for use in Bioelectric Systems.

For goal 1, a standardized biobrick backbone was selected and used in all four microbial chassis. For goal2, the initial aim was to create and clone four reporter modules (arsenic, lead, zinc and cadmium), but this aim has been expanded to include mercury, chromium and copper. With the exception of arsenic, the cadmium, chromium, copper, lead, mercury and zinc reporter modules have been cloned. For goal 3, the sensitivity of cadmium, mercury and lead biosensors were created and tested. For goal 4, the electroactive microbes were created with the development of overexpression constructs, growth of the respective strains and were tested.

This funding resulted in 13 peer reviewed published papers and numerous conference presentations.

15. SUBJECT TERMS
microbial biosensors, synthetic biology

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"**Synthetic Electric Microbial Biosensors**"

Date

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Abstract: Long-term continuous monitoring of heavy metals is required for early detection to reduce human exposure and accumulation to toxic levels in the environment. Synthetic biology has enabled the construction of molecular circuits with measurable outputs in response to environmental pollutants. This study is focused on utilising electroactive bacteria as biosensors. Redox-active proteins which are produced by these bacteria have specific electrochemical signals that can be detected using cyclic voltammetry. As a proof of principle in the development of integrated biosensors, genes encoding electroactive cytochromes have been cloned downstream of promoters and their cognate transcriptional regulators. These biosensors are being designed to generate electrochemical outputs in response to various heavy metal analytes. This study aims to develop whole-cell biosensors for heavy metals which can be incorporated into existing field-deployable sediment microbial fuel cells designed for environmental monitoring.

Introduction:

Heavy metals
Heavy metals are among the most abundant, toxic and persistent inorganic environmental pollutants. Long term continuous monitoring is required for early detection to reduce human exposure and accumulation to toxic levels in the environment [1]. In particular, monitoring of heavy metals in the environment, drinking water, food, and biological fluids is of interest. Conventional techniques for heavy metal detection require tedious sample preparation, preconcentration and laboratory based techniques such as atomic absorption spectroscopy, inductively coupled plasma/mass spectrometry, ultraviolet-visible spectroscopy amongst others [2].
Due to the continued industrial activity, instances of contamination, and the potential for deliberate spills, interest has grown in portable devices for onsite long-term detection using sensor technology [3, 4] Research is currently investigating the incorporation of biological material with optical, electrochemical and field-effect transistor sensors to develop multiple detection devices [1, 3]. Of particular interest are whole cell systems that can be electrically integrated into biosensor systems for the online detection of a range of contaminants.

Synthetic Biology and biosensors
Synthetic biology has gained much interest recently due the potential to construct and design molecular circuits in microbial chassis for desirable outputs including: environmental sensing; alternative energy solutions; production of pharmaceutical products; and targeted therapies for

novel treatment or tissue replacement strategies [5, 6]. An appealing aspect of synthetic biology is the modular nature of the genetic components, termed biobricks®, which can be assembled to carry out a desired process in a preprogramed fashion across different microbial chassis [7]. While the number of verified biobricks®, modular circuits and chassis organisms have been increasing; the application of synthetic biology is not straight forward [8]. This is in part due to the inherit complexity and dynamic conditions within living cells and across microbial species. A majority of synthetic biology constructs have utilized *E. coli* as a chassis but experience poor growth, toxicity of byproducts or incompatibility with the environments of interest [9].

In terms of biosensing, synthetic biology components need to be incorporated into robust environmentally relevant chassis with easily detectable outputs. In theory, electrode associated microbes have the potential to provide electrochemically defined outputs from defined environmental inputs providing electrically integrated microbial biosensors. A potential problem of relying solely on electrical output of an anode-associated microbe is the fluctuation in current production due to environmental conditions. Changes in temperature, nutrient content amongst others could all lead to variations of current production and sensory false signals. Therefore the objective of the work proposed herein describes the use of synthetic biology to produce alternative electrically detectable signals in a range of electrode associate microbes for continuous long term monitoring purposes.

Sensitivity and Specificity of Heavy Metal Promoters
Regulators that respond to heavy metal ions and metalloids are currently being extensively studied for there application as sensory elements in whole cell biosensors. Metalloregulators that incorporate metal binding sensor domains and DNA-binding domains into a single protein for deregulation of down stream genes of have been favored [10]. Initially experiments with monovalent responsive proteins found responded to different metal ions with the same charge and chemistry, such as ZntR binding of zinc (Zn(II)), cadmium (Cd(II)), or lead (Pb(II)) or CueR binding to copper (Cu(I)), silver (Ag(II)) or gold (Au(I)) [11]. Increase sensitivity and specificity has been achieved with many metalloregulators through selection and screening, site directed mutagenesis and motif swapping [11]. For instance Cd(II) specific biosensors have been developed that do not respond to zinc or lead [12].

In relation to this project the proposed the previously studied metalloregulators: *arsR* for arsenic [13]; *pbrR* for lead [14]; *zntA* for zinc [15]; and *yodA* (also referred to as *cadR*) for cadmium [16] have been selected as sensor modules.

Arsenic is a commonly found in the environment as either aresnite or arsine As(III) and arsenate As(V) which are highly toxic to living organisms. The current WHO and US Environmental Protection Agency recommended drinking water limit is 10 ug/L. The abundance of arsenic across a wide variety of environments has lead to the evolution of detoxification systems. The arsenic resistance system (*asr)* is reported to be the most widely distributed amongst prokaryotes with expression controlled by the transcriptional repressor ArsR [17]. The overexpression of ArsR with an elastin-like polypeptide fusion partner was found to specifically bind arsenic over higher concentrations of Cd and Zn [18].

Previous reports have place the expression of fluorescent proteins under the regulatory control of AsrR and found time dependent and dose dependent response to As^{3+} and As^{5+}[19]. Using whole cell biosensors, detection limits bellow 10 ug/L can be regularly achieved across a range of microbes such as *E. coli, B. subtilis* and *Staphylococcus*, within 30 mins to several hours [20, 21]. Reports have indicated that detection levels can be as low as 2.5 ug/L using *E. coli* [22]. Bioreporters based on *B. subtilis* and related organisms found similar detection levels [23]. One of the more successful synthetic biology derived biosensors is known at the "Edinburgh" pH-based arsenic biosensor in *E. coli* [6]. This acid generating system linked with a pH indicator gave reliable responses as low as 2.5 ppm and correlated well with arsenic levels in model groundwaters and also in real arsenic-contaminated groundwater samples from Hungary [20].

A cadmium biosensor developed in *E. coli* using *yodR (cadR)*, a cadmium responsive reporter fused to LacZ was able to detect concentrations as low as 25 nM in water and seawater as well as 5 uM in untreated soil. These detection levels are below the EPA maximum contaminant goal 5ug/L. Within this system no regulation of the *yod* system was observed when challenged with zinc, copper, cobalt and nickel [24]. Truncation of CadR by either 10 or 21 aminoacids also lead to an increased specificity of Cd(II) over Zn(II) or Hg (II) by reducing the induction coefficient for these metals by up to 95% [25]. A Fluorescent based biosensors in *E. coli* also reported in a proof of concept test detection limits of 0.5 uM for Cd(II) while using multiple strains to also detect As(II) in a multi-channel sensor system [26]. Colorimetric sensor systems for cadmium has also been developed in *Deinococcus radurans* and *B. subtilis* have reported similar low detection limits [5].

While potentially toxic heavy metals within the environment, biosensor systems for lead and zinc have not been as fully explored as arsenic and cadmium, likely due to their listing on the secondary drinking water guidelines and with initial problems with specificity being less then those previously mentioned. Previously zinc biosensor systems were reported to cross-react with other heavy metals in specific Cd(II) and Pd(II). Single amino acid substitutions have have been successfully applied to change the relative location of metal-binding cysteine residues and change the response profile of ZntR to Zn(II), Cd(II) and Pb(II). More recently a zinc specific biosensor was developed in *P. putida* X4 and *Synechocystis* sp. utilizing a *zntR* homologs able to detect 90% of the zinc concentrations within water and soil samples, with detection limits as low as 1-3 uM/L without cross reactivity in *P. putida* X4 from Cd, (II) Cu(II) and Co(II) [27]. This detection limit is below the EPA national secondary drinking water guidelines of 5mg/L. Since this is the first reported zinc specific reporter then screening is expected to increase those available of use in synthetic biology produced biosensors.

Lead (Pb(II)) specific biosensors have also been previously hampered by cross reactivity with Cd (II) but research and development of specific response regulators progressed similar to Zn (II). The BIOMET sensor system utilizes a range of sensor including Pb(II) in the host strain *Ralstonia metallidurans* CH34 with detection limits as low as 0.08 uM. The bioavailability of Pb(II) after bioremediation treatment with phosphate, lime or ash with the Pb(II) BIOMET microbial biosensor successfully determined bioavailability profiles across these treatments [28]. The BIOMET biosensors were also able to distinguish the bioavailability of a variety of heavy metals without cross-reactivity between Cu, Zn, As, Pb in soil samples [29].

The influence and affect of ions in samples has not been extensively studied on biosensors as most have been utilized in the testing of ground waters. From the information available, as reported above, the presence of other metals, such as copper, manganese or iron has not specifically effected expression of the reporters. Tests of 112 water samples from a wide range of highly variable ground water samples reported only 8% of samples gave false negatives and 2.4% gave false negatives in test for arsenic ustilizing the *asrR* system [30]. Similarly previous studies have demonstrated that *asrR* was not effected by the presence of Cd(III), Pb (II) [31] nor by elevated NaCl concentrations [18]. Interestingly the presence of bicarbonate in some case increased the sensitivity of response [22]. A Pb biosensor based on *pbrR* also reported no affect from increasing ion concentrations of Zn, Ca, Cd, Hg, Al, Co, Se, Wo and As [32]. While not specifically addresses, similar reporters do not report that specific increases in other ions such as sodium, iron, copper, manganese or copper in soil samples did not significantly effect the responsive range of the biosensor systems. The effect of marine and brackish waters will be addressed within this research proposal.

Project Objectives:

- To develop an electrically integrated microbial biosensor using model electroactive microbes through synthetic biology
- To evaluate standard promoter-based sensor inputs and outputs in electroactive microbes
- To develop electrically detectable signal outputs in electric microbes
- To combine sensor inputs and electrical outputs in electroactive microbes for use in Bioelectric Systems

Technical Approach:

- Synthetic biology: Development of biobricks for use in *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PAO1, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA
- Expression of redox-active *c*-type cytochromes
- Detection of redox-active compounds by cyclic voltammetry and Raman spectroscopy
- Integration into Bioelectric Systems to allow real-time monitoring

Experiment: Please see attached manuscripts

Summary of Results:

Task 1: A standardized biobrick backbone has been selected for rapid interchange of input modules (responsive to the presence of heavy metal ions) and output modules (produce a fluorescent or an electrochemical signal). The suitability of the biobrick backbone has been assessed in *Escherichia coli*, *Pseudomonas aeruginosa*, *Shewanella oneidensis* and *Geobacter sulfurreducens*. The backbone can stably exist in all four microbial chassis, can be easily transferred between strains and allows expression of the reporter proteins (output modules). The four microbial chassis have been cultured aerobically and anaerobically in various media to assess their optimal growth conditions. Also their resistance to various antimicrobial agents was analysed. See Figure 1-3.

Task 2: The initial aim was to create and clone four reporter modules (arsenic, lead, zinc and cadmium), but this aim has been expanded to include mercury, chromium and copper. With the exception of arsenic, the cadmium, chromium, copper, lead, mercury and zinc reporter modules have been cloned. See Figure 4.

Task 3: The sensitivity and specificity of cadmium, mercury and lead biosensors have been tested. Mercury and cadmium biosensors constructed in this study can detect Hg^{2+} and Cd^{2+} within minutes at low levels and are highly specific. The guideline value of WHO for inorganic mercury (Hg^{2+}) in drinking water is 0.006 µg ml^{-1}, *E. coli* and *P. aeruginosa* mercury biosensors can detect 0.002 µg ml^{-1} following 4 hours of exposure. The presence of Hg^{2+} is detected within 5-20 minutes following exposure depending on the strain. Mercury biosensors can only detect Hg^{2+} and not other heavy metals at concentrations up to 0.1 µg ml^{-1}. The lead biosensor can detect Pb^{2+}, however it does so only at moderate levels (two-fold above the WHO acceptable limits) and can also detect a range of other heavy metals. See Figure 5-19 and Table 1.

Task 4: OmcF, OmcS and OmcZ *c*-type cytochromes overexpressing constructs have been constructed as well as the riboflavin overexpressing construct. A plasmid carrying the cytochrome *c* maturation operon (*ccm*) which is regulated by a constitutive *tet* promoter has been introduced into all the strains carrying the *c*-type cytochromes overexpressing constructs to ensure maturation of OmcF, OmcS and OmcZ proteins. See Figure 20.

Task 5: Growth of the overexpressing *c*-type cytochrome strains has been assessed. The protocol for isolation of insoluble proteins has been developed.

Task 7: Several electrochemical biosensors have been constructed: cadmium-inducible OmcS, OmcF and Bfe constructs as well as mercury-inducible OmcS, OmcZ and Bfe constructs. See Figure 21.

Note: Task 6 and Task 8 involve the testing of the electrochemical signal of overexpressing cytochromes as well as the electrochemical biosensors via electrode analysis. Therefore, these tasks require the completion of the preceding Tasks before they can be commenced.

Detailed Results:
Task 1. Development and evaluation of biobrick backbone in *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PAO1, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* DL-1. The suitability of the biobrick backbone has been assessed in the microbial chassis. The four microbial chassis have been cultured aerobically and anaerobically in various media to assess their optimal growth conditions. Also their resistance to various antimicrobial agents was analysed in order to select a suitable biobrick backbone.
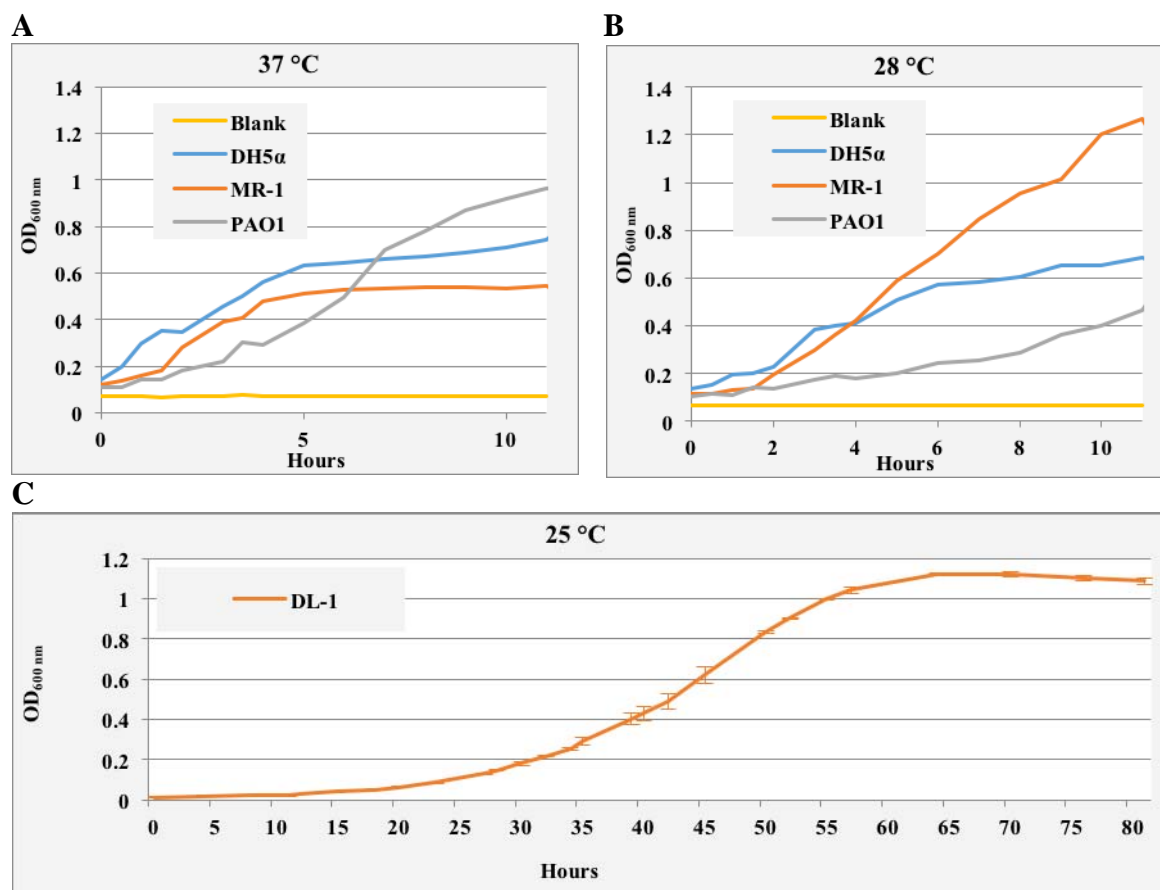
Figure 1. Growth curves of Luria broth cultures of *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PAO1, *Shewanella oneidensis* MR-1 incubated aerobically at **(A)** 37 °C and **(B)** 28 °C. **(C)** Growth curves of NBAF-culture of *Geobacter sulfurreducens* DL-1 incubated anaerobically at 25 °C.

A suitable biobrick backbone was chosen: (i) could be selected for using antimicrobial agents, (ii)

could be easily transferred via conjugation, (iii) could exist stably, and (iv) allows expression of the reporter protein (green fluorescent protein, GFP).
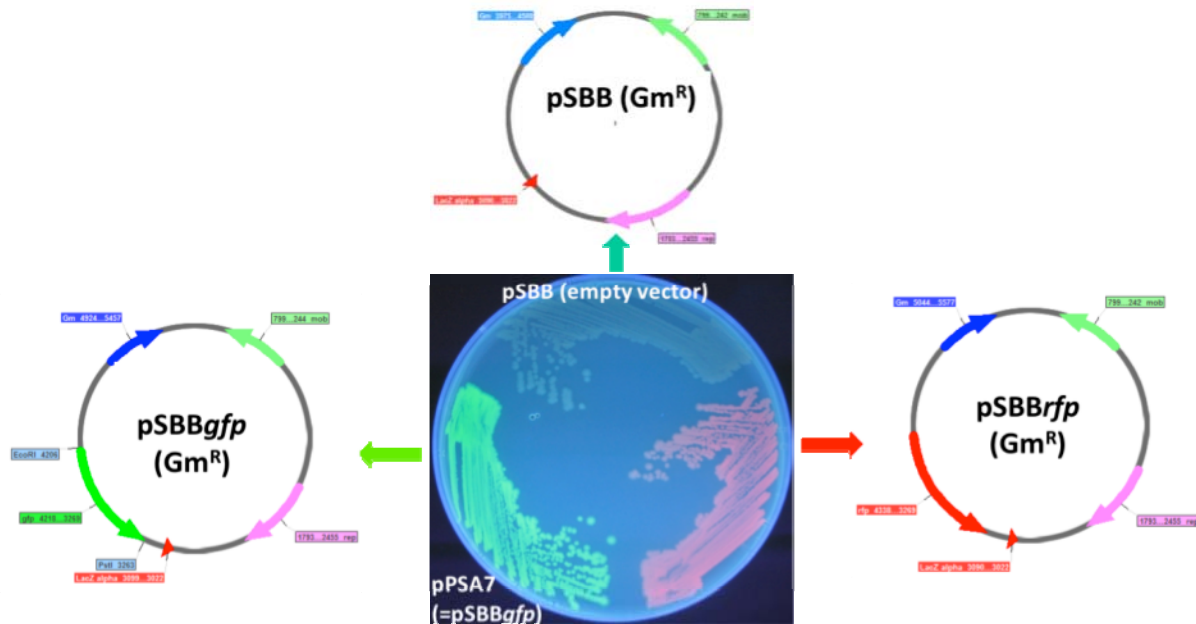

Figure 2. *E. coli* carrying pSBB, pSBB*gfp* and pSBB*rfp* on LA+Gm$_{10}$ incubated overnight at 37 °C and photographed under UV-light. The GFP and RFP reporters are constitutively expressed from their native promoters.

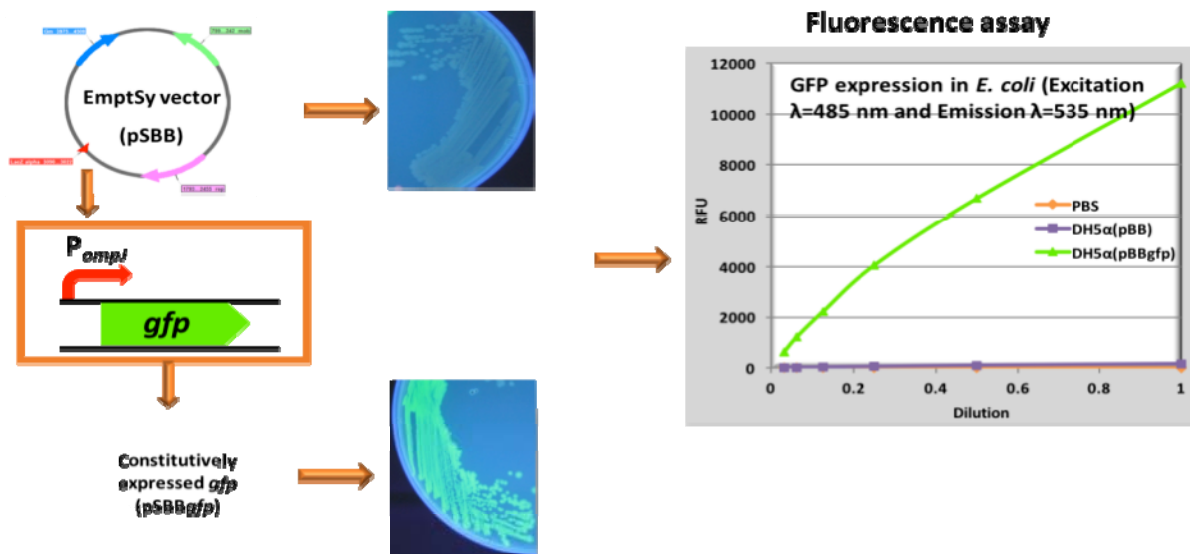**Figure 3.** Promoterless *gfp* gene has been cloned downstream of a constitutively expressed *ompJ* promoter (P$_{ompJ}$). *E. coli* carrying pSBB and pSBB*gfp* on LA+Gm$_{10}$ incubated overnight at 37 °C and photographed under UV-light. Fluorescence assay of the constitutively expressed GFP. The constitutively expressed promoter will be used for expression of cytochromes in Task 4.

Milestone: A suitable biobrick backbone has been chosen for the construction of input and output modules.
Task 2: Cadmium, chromium, copper, lead, mercury and zinc modules have been cloned. The initial proposal was to construct four sensor modules (arsenic, cadmium, lead and zinc), however

this has been expanded to include chromium, copper and mercury.
In each instances the regulatory gene and the heavy-metal inducible promoter were
synthesized prior to being cloned into the biobrick backbone.
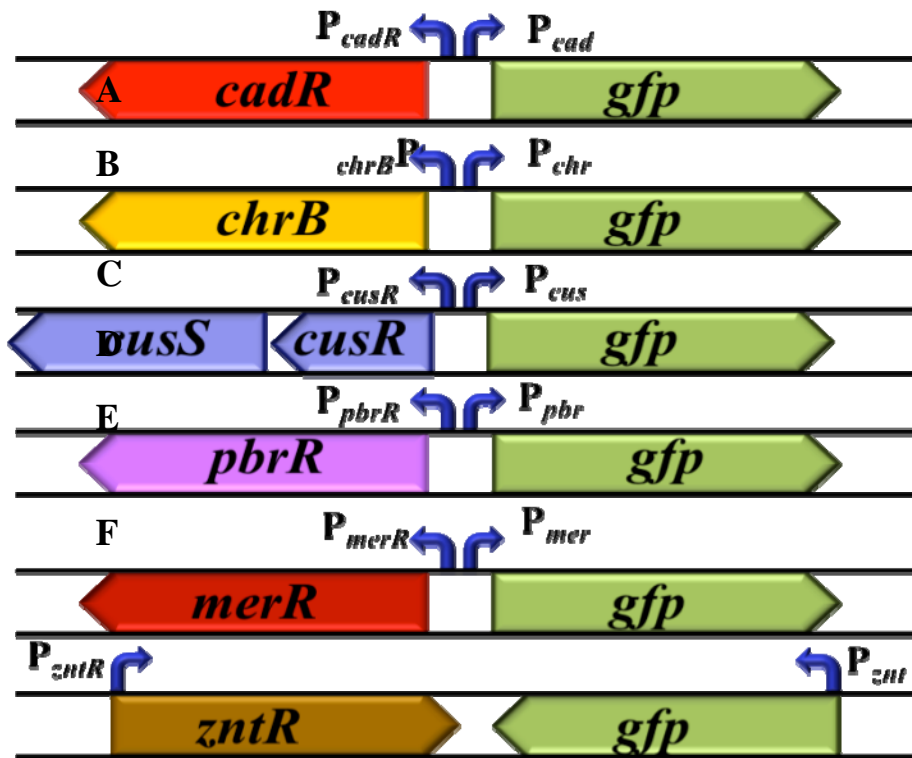
Figure 4. A schematic diagram of the six cloned reporter modules. The promoterless *gfp* gene
(731 bp) in each instance has been cloned from a biobrick constitutively expressing GFP
(iGEM, BBa_I13522).
(A) A cadmium transcriptional regulator (CadR) along with the promoter is from *Pseudomonas
putida* 06909 (NCBI Accession no. AF333961).
(B) The chromium transcriptional regulator (ChrB) and the promoter it regulates is from a
Ochrobactrum tritici transposon TnOtChr (NBCI Accession no. EF469735).
(C) A sensory histidine kinase (CusS) and Copper transcriptional regulator (CusR) along with
the promoter is from *E. coli* K12 (NCBI Accession no. AF245661).
(D) A lead transcriptional regulator (PbrR) along with the promoter is from
Klebsiella pneumoniae KCTC 2242 plasmid pKCTC2242 (NCBI Accession no.
CP002911.1).
(E) The gene encoding the mercury transcriptional regulator (MerR) and the promoter it
regulates was cloned from *Bordetella bronchiseptica* plasmid R906 (NCBI Accession no.
KF743818).
(F) A zinc transcriptional regulator (ZntR) along with the promoter is from *Escherichia coli*
PCN061 (NCBI Accession no. CP006636.1).

Milestone: Creation of cadmium, chromium, copper, lead, mercury and zinc biosensors. Task 3: The sensitivity and specificity of cadmium and mercury biosensors has been tested.

Task 3: Qualitative assessment of *Escherichia coli* mercury and cadmium biosensors on agar plates.
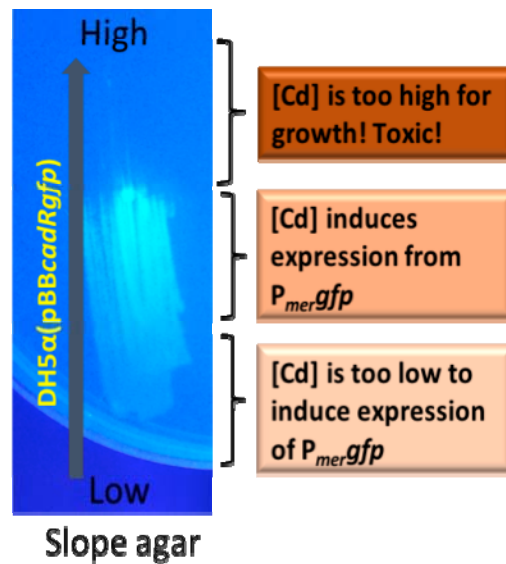
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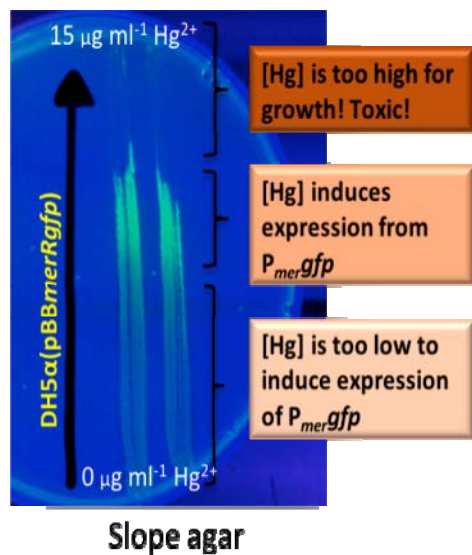
B

Figure 5. A photograph of a **(A)** mercury and **(B)** cadmium biosensor in *Escherichia coli* on agar slope Luria Bertani medium containing mercury (Hg^{2+}) and cadmium (Cd^{2+}), respectively, exposed to UV light. Heavy metal ions are highly toxic, which is evident from the lack of growth at the top of the plate. At the bottom of the plate, where there is no heavy metal ions present, there is no fluorescence. The expression of *gfp* is only induced in the presence of heavy metal ions. This is a qualitative assessment that can be used as a preliminary test for the remaining biosensors.
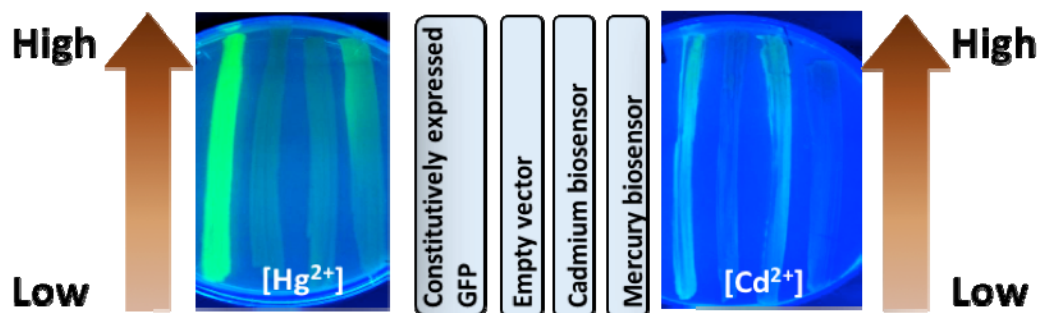

Figure 6. Cadmium and mercury biosensors on slope agar plates containing increasing concentrations of mercuric ions (Hg^{2+}) and cadmium ions (Cd^{2+}). Constitutively expressed GFP

strain produces a fluorescent signal regardless of the presence of a heavy metal whereas the strain carrying the empty vector produces no fluorescent signal. Increasing concentration of heavy metal
ions induce expression of GFP in the biosensors; mercury biosensor produces a fluorescent signal only following induction with Hg^{2+} and cadmium biosensor produces a fluorescent signal only following induction with Cd^{2+}.

Task 3: Qualitative assessment of *Escherichia coli* mercury biosensor in liquid culture.

A
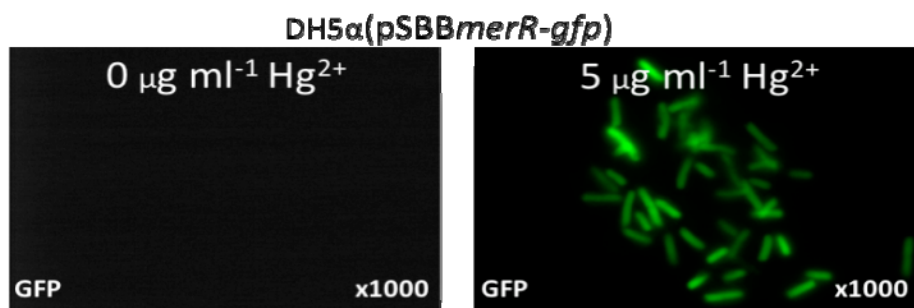
B
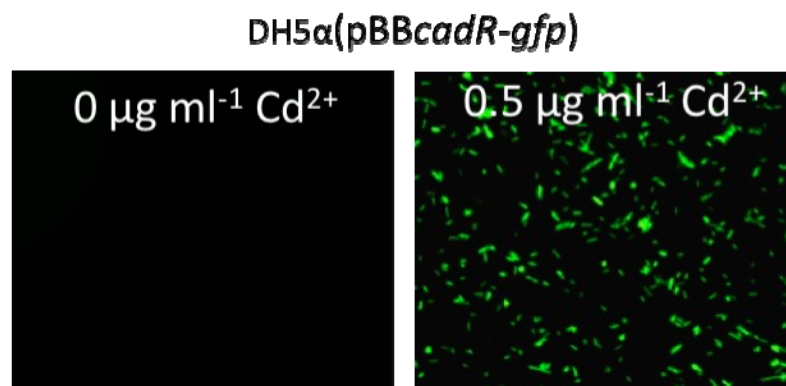
Figure 7. Fluorescence microscopy (GFP-filter) images of (A) mercury and (B) cadmium biosensors (heavy metal-inducible *gfp*) in *Escherichia coli*, induced (left image) and uninduced (right image) with 5 µg ml^{-1} Hg^{2+} and Cd^{2+} for 4 hours. The culture was grown in Luria-Bertani broth to exponential phase prior to induction with heavy metal ions.

Task 3: Qualitative assessment of the expression of the reporter protein (GFP) in the *Escherichia coli* mercury biosensor.
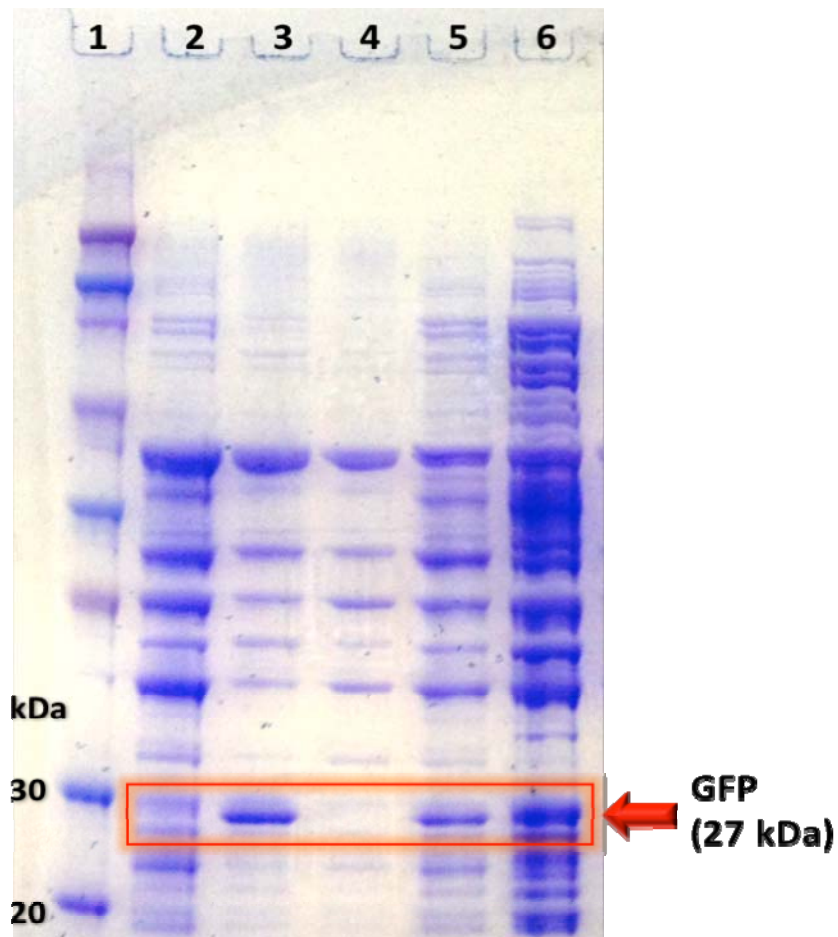
Lanes:
1. Ladder
2. Empty vector
3. Constitutively expressed *gfp*
4. Uninduced mercury biosensor
5. Induced (1 µg ml^{-1} Hg^{2+}) mercury biosensor
6. Induced (5 µg ml^{-1} Hg^{2+}) mercury biosensor

Figure 8. SDS-PAGE of total soluble protein of *Escherichia coli* strains carrying the biobrick backbone, the constitutively expressed *gfp* and the mercury biosensor construct.
GFP is 27 kDa and is only evident in Lane 3 (constitutively expressed GFP), Lane 5 and Lane 6 (mercury biosensor induced with 1 and 5 µg ml^{-1}, respectively).
This is a qualitative assessment of the expression of the reporter protein, GFP, which will later be used to assess the expression of the cytochromes in Task 5.

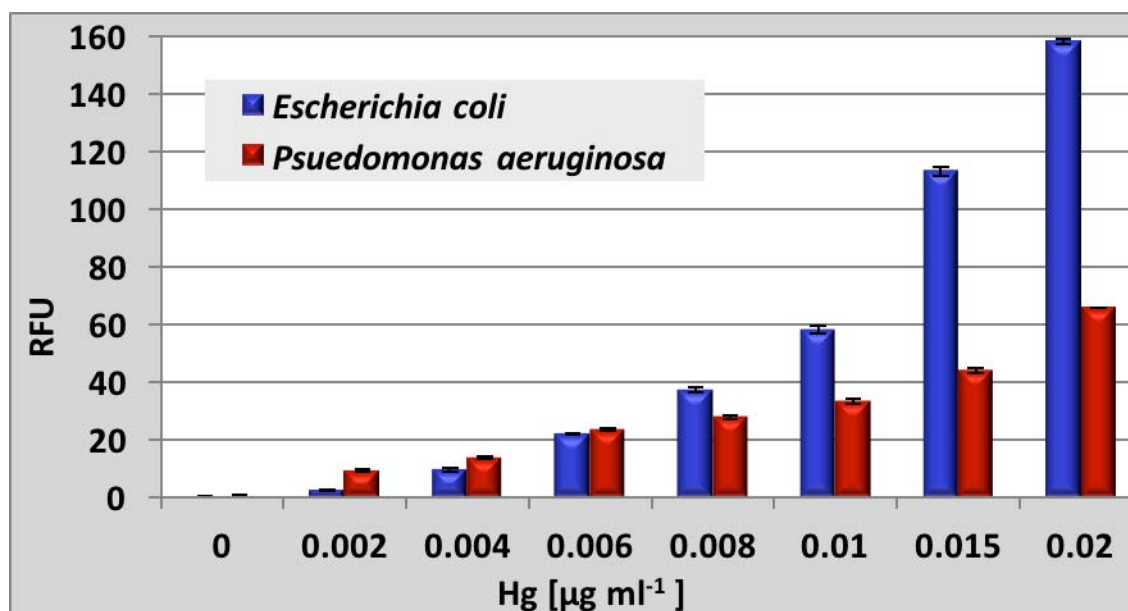**Task 3:** Sensitivity testing of the mercury biosensors using fluorescence assays

Figure 9. *Escherichia coli* and *Pseudomonas aeruginosa* mercury biosensors are highly sensitive. The guideline value of WHO for inorganic mercury (Hg^{2+}) in drinking water is 0.006 µg ml^{-1} The sensitivity of the biosensors is dependent on the exposure time *E. coli* and *P. aeruginosa* mercury biosensors induced with Hg^{2+} (0-0.02 µg ml^{-1}) for 4 hours
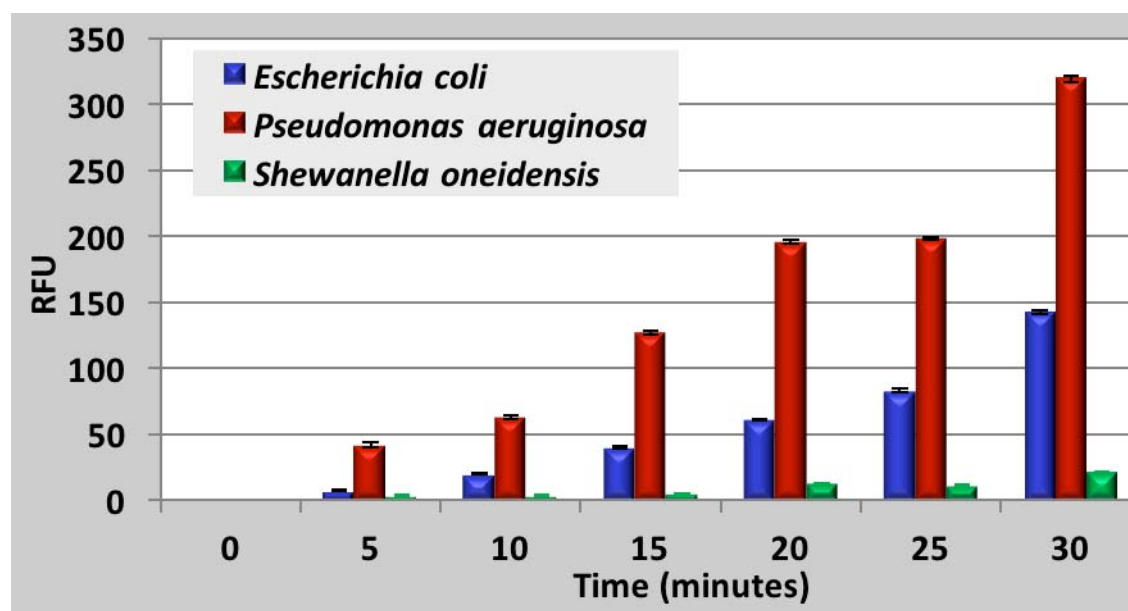

Figure 10. *Escherichia coli*, *Pseudomonas aeruginosa* and *Shewanella oneidensis* mercury biosensors can detect mercury within 5-20 minutes of exposure. *E. coli*, *P. aeruginosa* and *S. oneidensis* mercury biosensors induced with 0.1 µg ml^{-1} of Hg^{2+} over 30 minutes. The fluorescence signal increased over time. The time taken to detect the presence of Hg^{2+} is dependent on the concentration of Hg^{2+}
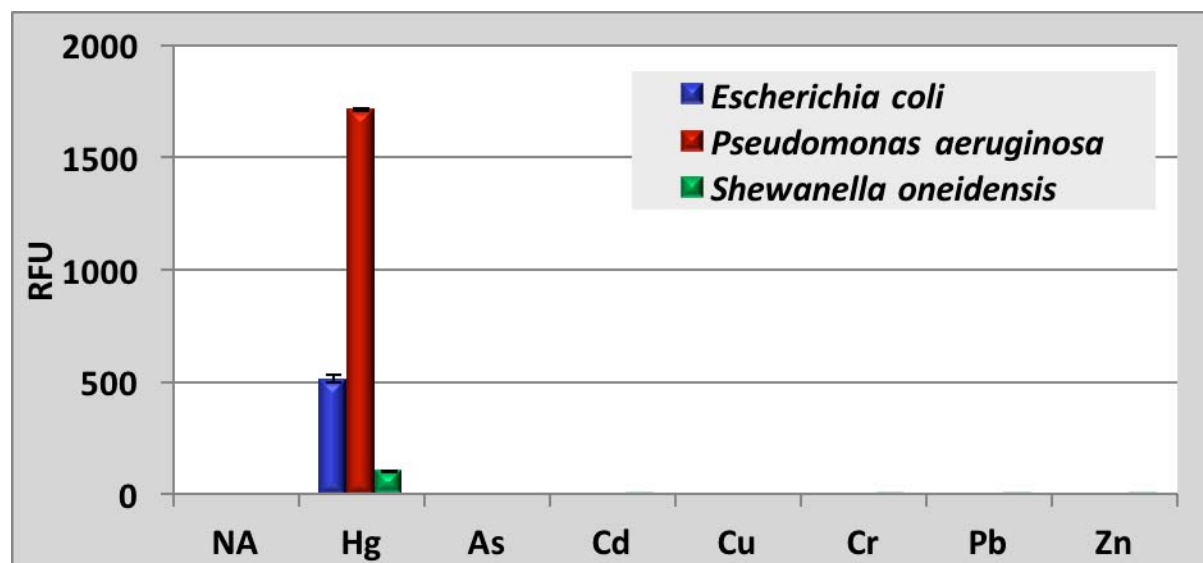
Figure 11. *E. coli*, *P. aeruginosa* and *S. oneidensis* mercury biosensors are highly specific for mercury. The cultures were uninduced (NA) and induced with Hg^{2+} and other heavy metals (0.1 µg ml^{-1}) for 4 hours.

Varying concentrations of heavy metals (arsenic, cadmium, chromium, copper, lead, mercury and zinc) were used in specificity testing of the fluorescent biosensors. The fluorescence was monitored at various concentrations of heavy metal ions to determine the sensitivity and specificity of each construct so specific heavy metal ion.

Fluorescence assays are dependent on: the composition of the culture medium (nutrient-rich or nutrient-poor), growth phase of the induced bacterial culture, the final OD of the exposed culture, the exposure time and concentration of the heavy metal ions.

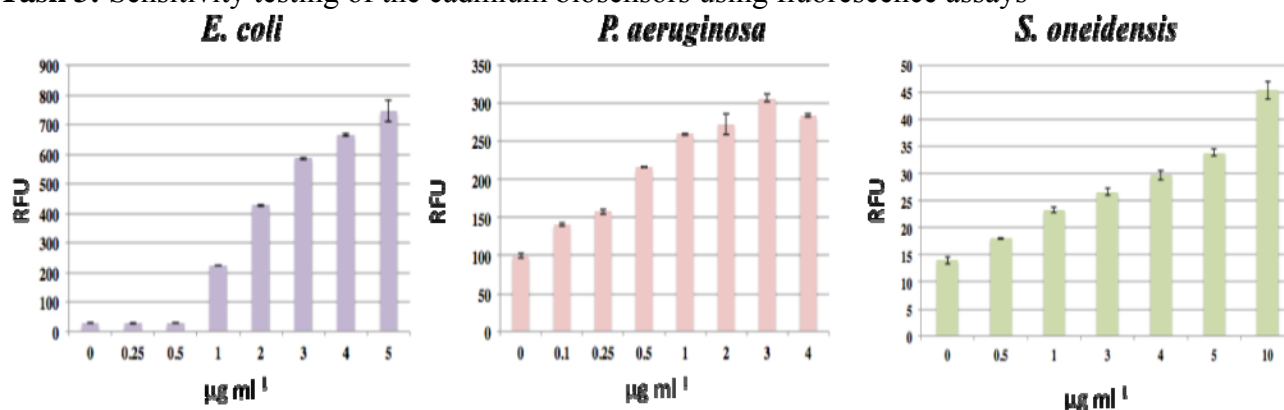**Task 3:** Sensitivity testing of the cadmium biosensors using fluorescence assays

Figure 12. Sensitivity assays of *Escherichia coli*, *Pseudomonas aeruginosa* and Shewanella *oneidensis* cadmium biosensors.
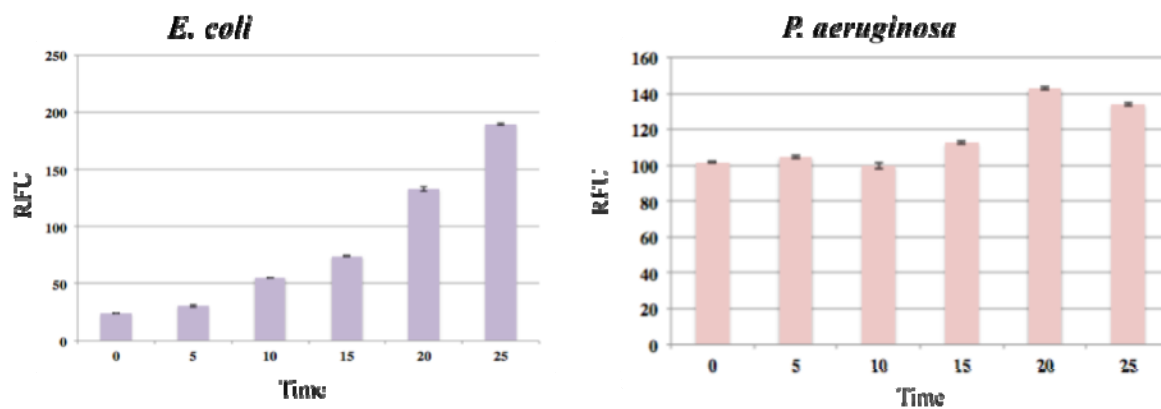
Figure 13. *E. coli* and *P. aeruginosa* cadmium biosensors induced with 2 μg ml^{-1} of Hg^{2+} over 25 minutes
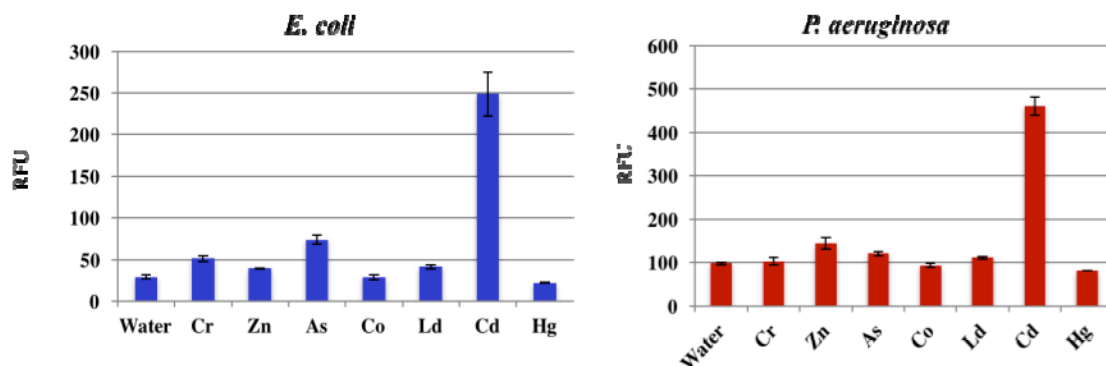
Figure 14. Specificity fluorescence assays: Cadmium biosensors are highly specific for high concentrations of cadmium
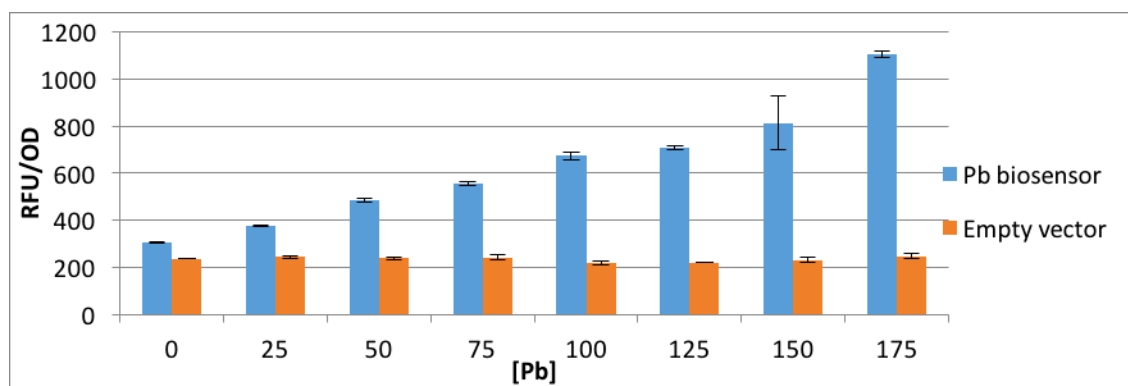
Figure 15. Sensitivity assays of *Escherichia coli* lead biosensor and the same strain containing the empty vector (negative control). The strains were exposed to various concentrations of Pb^{2+} for 4 hours. The biosensor can detect 25 µg ml^{-1} Pb^{2+}.
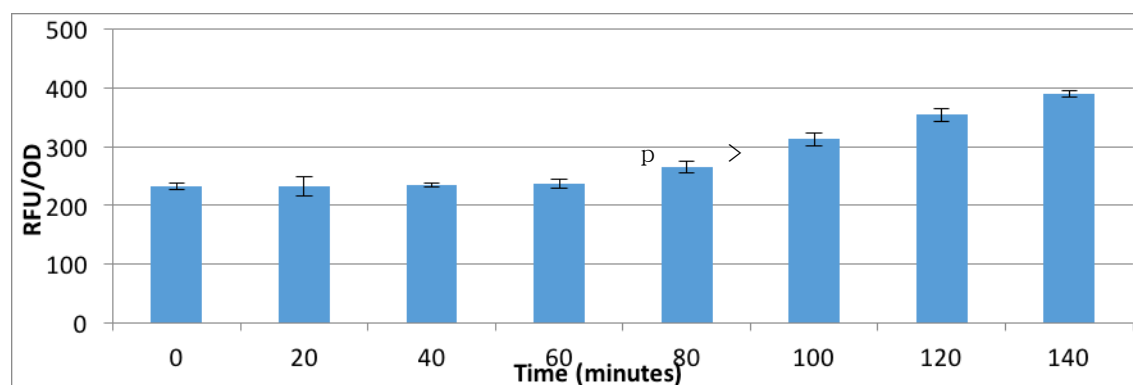

Figure 16. The *Escherichia coli* lead biosensor can detect 100 µg ml^{-1} Pb^{2+} following 80 minutes of exposure.
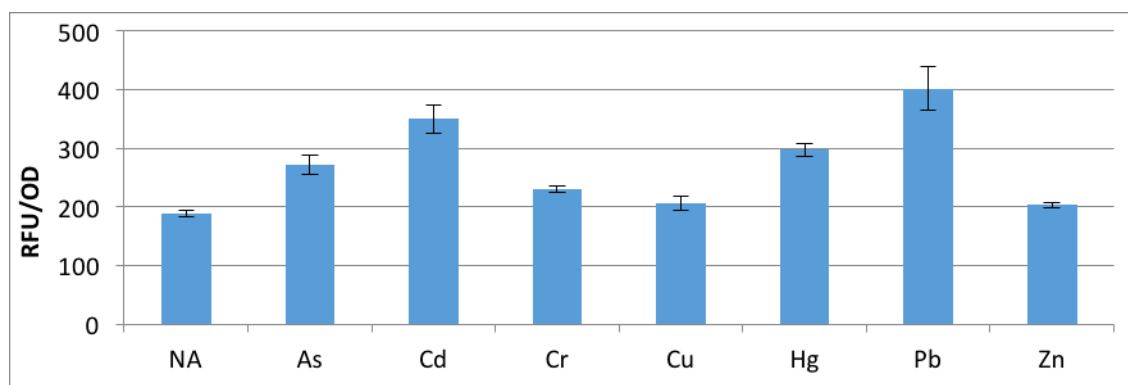

Figure 17. The *Escherichia coli* lead biosensor can detect 100 µg ml^{-1} Pb^{2+}, As^{3+}, Cd^{2+}, Cr^{5+} and Hg^{2+} following 4 hours.

Milestone: Mercury and cadmium biosensors constructed in this study can detect Hg^{2+} and Cd^{2+} within minutes at low levels and are highly specific. The lead biosensor can detect a number of heavy metals at moderate levels.

Task 3: Analysis of microbial chassis in ocean water and response to various heavy metals.
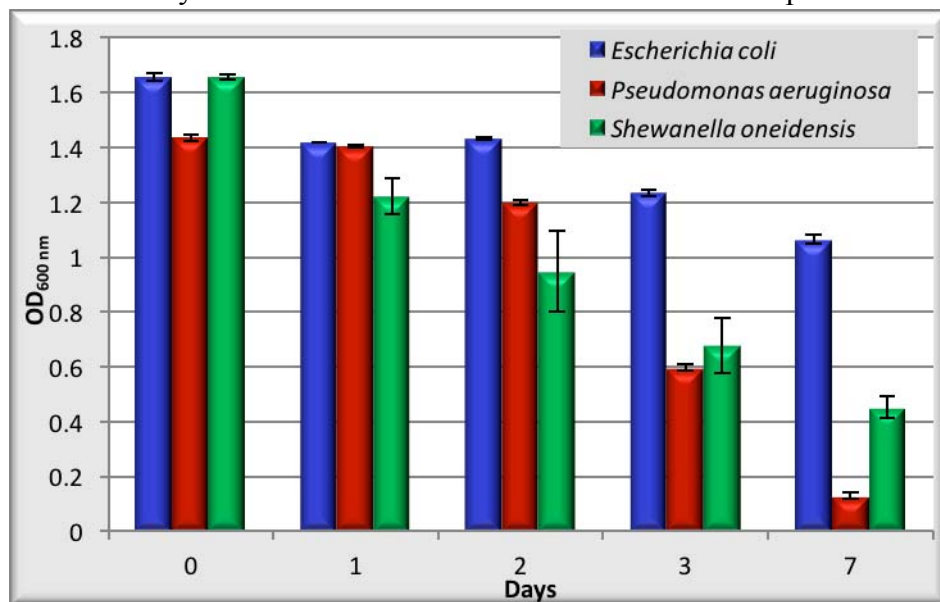

Figure 18. Growth of *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PAO1 and *Shewanella oneidensis* MR-1 in ocean water over 7 days. The microorganisms can survive for 7 days in ocean water.
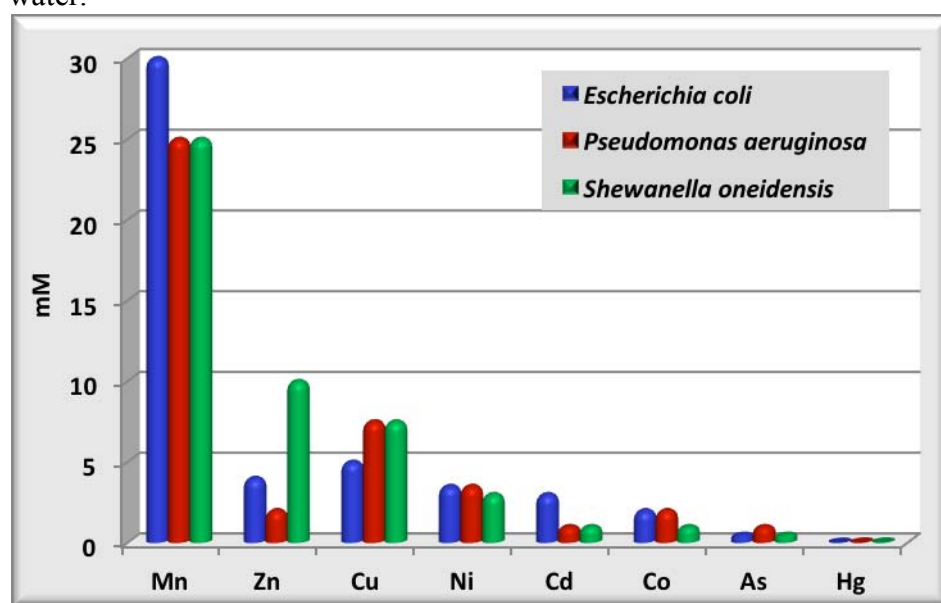

Figure 19. Minimum inhibitory concentrations (mM) of common marine metals and contaminants (manganese, zinc, copper, nickel, cadmium, cobalt, arsenic and mercury).

Table 1. Minimum inhibitory concentrations (mM) of common marine metals and contaminants (manganese, zinc, copper, nickel, cadmium, cobalt, arsenic and mercury).

	Minimum Inhibitory Concentration (mM)							
	Mn	**Zn**	**Cu**	**Ni**	**Cd**	**Co**	**As**	**Hg**
Escherichia coli	30	4	5	3.5	3	2	0.5	0.022
Pseudomonas	25	2	7.5	3.5	1	2	1	0.037
Shewanella	25	10	7.5	3	1	1	0.5	0.044

Task 4: Four out of five *c*-type cytochromes overexpressing constructs have been made. In the initial proposal only 4 electroactive proteins were going to be cloned, this has been expanded to include OmcZ. The *ompJ* constitutive promoter has been cloned for overexpression of *c*-type cytochromes. The functionality of the promoter has been confirmed by the fusion of a

promoterless *gfp* gene (see Figure 3). OmcF, OmcS, OmcZ and the bacterial flavin adenine dinucleotide exporter (*bfe*) have been fused to the *ompJ* constitutive promoter. The cloning of OmcB is in progress. Protein assays have been conduced to detect the presence of insoluble proteins, however this will require further troubleshooting as *Escherichia coli* already expressed insoluble proteins of similar size, therefore in order to differentiate will need to perform heme staining.
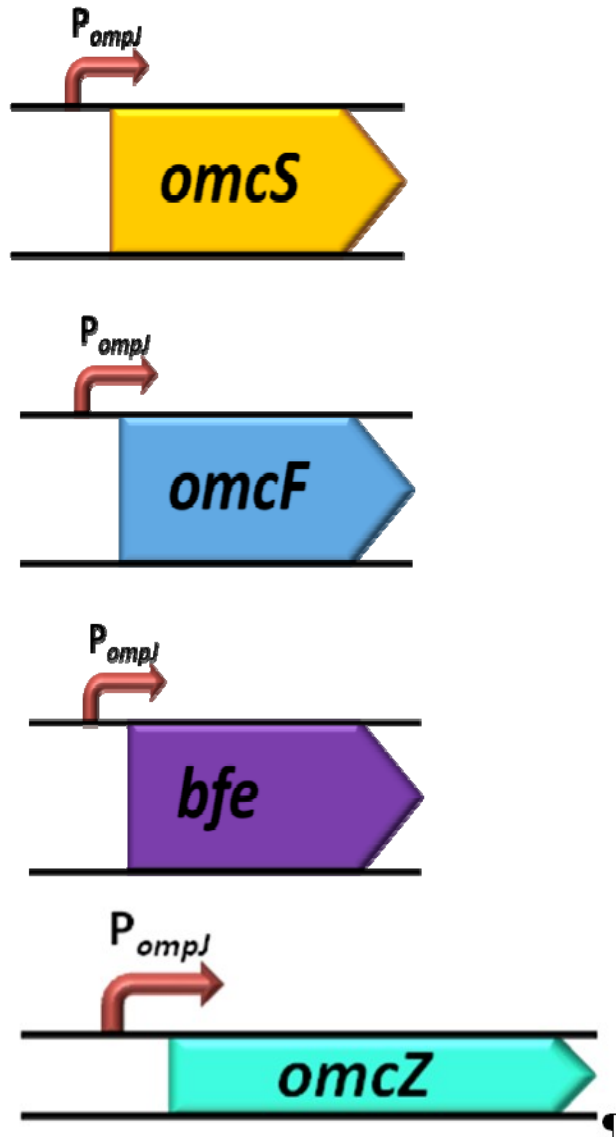

Figure 20. The outer membrane cytochrome OmcS, OmcF, OmcZ as well as the bacterial flavins adenine dinucleotide exporter (bfe) have been cloned downstream of the constitutive *ompJ* promoter (P*ompJ*).
Milestone: Four out of five strains overexpressing cytochromes and riboflavin have been constructed.
Task 5: Growth of the overexpressing *c*-type cytochrome strains has been assessed. Polyacrylamide gel electrophoresis and heme staining is being done to determine the cytochrome protein content of the engineered strains compared to the wild-type.
Milestone: The protocol for isolation of insoluble proteins has been established.

Task 7: Several electrochemical biosensors have been constructed. Sensor systems from Task 2 have been used to control the expression of cytochromes from Task 4 in the biobrick backbone

developed in Task 1. These constructs will allow synthetically-derived detection of heavy metal ions (input signal) with the production of a specific *c*-type cytochrome or riboflavin (an output signal).
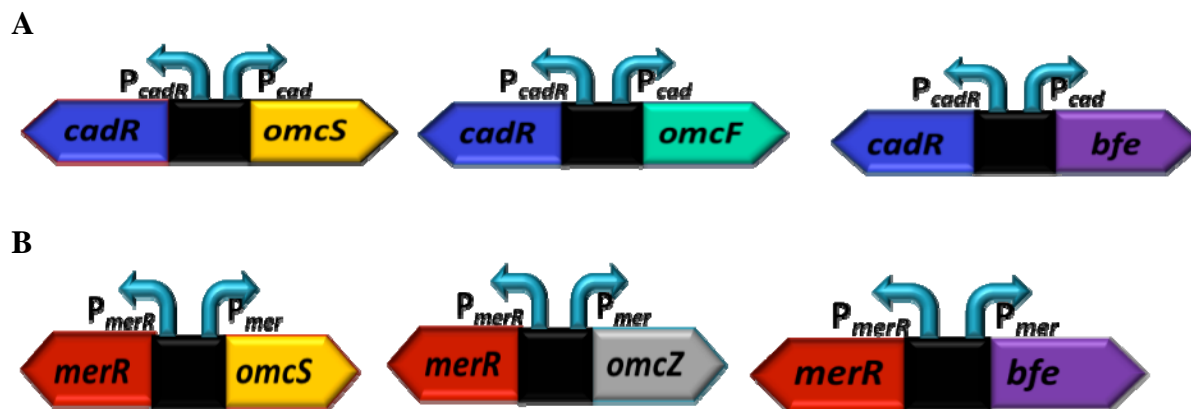
A

B

Figure 21. Modular biosensors which have been constructed.

Milestone: Cadmium-inducible OmcS, OmcF and Bfe constructs as well as mercury-inducible OmcS, OmcZ and Bfe constructs have been created.

List of Publications and Significant Collaborations that resulted from your AOARD supported project: In standard format showing authors, title, journal, issue, pages, and date, for each category list the following:

a) papers published in peer-reviewed journals,

1. Bell, Carolyn A, and Ashley E Franks (2017) Plugging in microbial metabolism for industrial applications. *Microbiology Australia.* 38(**2**):89-92.
2. Bereza-Malcolm, Lara, Sanja Aracic, Ruban Kannan, Gülay Mann, and Ashley E Franks (2017) Functional characterization of gram-negative bacteria from different genera as multiplex cadmium biosensors. *Biosensors and Bioelectronics.* 94:380-387.
3. Bereza-Malcolm, Lara, Sanja Aracic, and Ashley E Franks (2016) Development and application of a synthetically-derived lead biosensor construct for use in gram-negative bacteria. *Sensors.* 16(**12**):2174.
4. Michael, Carolyn A, Ashley E Franks, and Maurizio Labbate (2016) The antimicrobial resistance crisis: Management through gene monitoring. *Open Biology.* 6(**11**):160236.
5. Sivasubramaniam, Diane, and Ashley E Franks (2016) Bioengineering microbial communities: Their potential to help, hinder and disgust. *Bioengineered.* 7(**3**):137-144.
6. Wood, Jennifer L, Wuxing Liu, Caixian Tang, and Ashley E Franks (2016) Microorganisms in heavy metal bioremediation: Strategies for applying microbial-community engineering to remediate soils. *Soil Biology & Biochemistry.* 7:8.
7. Wood, Jennifer L, Caixian Tang, and Ashley E Franks (2016) Microbial associated plant growth and heavy metal accumulation to improve phytoextraction of contaminated soils. *Soil Biology and Biochemistry.* 103:131-137.
8. Wood, JL, C Zhang, ER Mathews, C Tang, and AE Franks (2016) Microbial community dynamics in the rhizosphere of a cadmium hyper-accumulator. *Scientific reports.* 6.
9. Aracic, Sanja, Sam Manna, Steve Petrovski, Jennifer L Wiltshire, Gülay Mann, and Ashley E Franks (2015) Innovative biological approaches for monitoring and improving water quality. *Frontiers in microbiology.* 6.

10. Bereza-Malcolm, Lara T, and Ashley E Franks (2015) Coupling anaerobic bacteria and microbial fuel cells as whole-cell environmental biosensors. *Microbiology Australia.* 36(**3**):129-132.
11. Semenec, Lucie, and Ashley E Franks (2015) Delving through electrogenic biofilms: From anodes to cathodes to microbes.
12. Aracic, Sanja, Lucie Semenec, and Ashley E Franks (2014) Investigating microbial activities of electrode-associated microorganisms in real-time. *Frontiers in microbiology.* 5:663.
13. Bereza-Malcolm, Lara Tess, Gülay Mann, and Ashley Edwin Franks (2014) Environmental sensing of heavy metals through whole cell microbial biosensors: A synthetic biology approach. *ACS synthetic biology.* 4(**5**):535-546.

b) papers published in peer-reviewed conference proceedings, S Aracic, G Mann, AE Franks Development of microbial biosensors using electroactive microbes for detection of hazardous analytes in the environment. Lorne Conference: Synthetic Biology and Protein Engineering. WEHI, Melbourne 6.

c) papers published in non-peer-reviewed journals and conference proceedings,

d) conference presentations without papers,

July, 2016 Rapid fire talk at SEED2016: Synthetic Biology, Engineering, Evolution and Design
- Conference presentation
- Title: "Development of a multiplexed cadmium biosensor for the detection of heavy metals in the environment"

July, 2016 Poster presentation at SEED2016: Synthetic Biology, Engineering, Evolution and Design
- Conference presentation

- Title: "Development of a multiplexed cadmium biosensor for the detection of heavy metals in the environment"

May, 2016 Highlighted Speaker to the Australian National Fabrication Facility- Air Force Office of Scientific Research (ANFF-AFOSR)
- Conference presentation
- Title: "Synthetically derived biosensors for heavy metal detection"

April, 2016 Highlighted PhD presentation at the inaugural Defence Science Institute (DSI) symposium
- Conference presentation
- Title: "Synthetically derived biosensors for heavy metal detection"

April, 2016 Poster presentation at the Synthetic Biology Cutting Edge Symposium
- Poster presentation
- Title: "Development of a multiplexed cadmium biosensor for the detection of heavy metals in the environment"

August, 2015 Oral presentation to the Department of Physiology, Anatomy and Microbiology, La Trobe University, Bundoora, Victoria 3086
- Departmental presentation
- Title: "Synthetically derived biosensors for heavy metal detection"   July, 2015   Poster presentation at the Australian Society of Microbiology (ASM) annual scientific meeting
- Oral presentation at the Australian Society of Microbiology (ASM) annual scientific meeting in Melbourne, Australia (3 day conference)
- Poster title: "Synthetically derived biosensors for heavy metal detection"

March, 2015   Invited speaker for graduate researchers
- Invited speaker to the 2015 orientation for graduate researchers, organised by the Research, Education and Development (RED) unit
- Talk title – "How to survive and thrive"

February, 2015   Poster presentation at the Lorne satellite conference: synthetic biology and protein engineering
- Poster presentation at Lorne satellite conference: synthetic biology and protein engineering in Melbourne, Australia (1 day conference)
- Poster title: "Anaerobic whole cell microbial biosensors"

October, 2014 Invited speaker at the16th EMBL PhD symposium (3 day international conference)
- Oral presentation at the 16th EMBL PhD Symposium entitled "Inspired by biology: Exploring natures toolbox" in Heidelberg, Germany

- Talk title: "Synthetic biology based microbial biosensors for the detection of heavy metals: a synthetic biology approach"
- Link: http://phdsymposium.embl.org/#home

October, 2014   Poster presentation at the 16th EMBL PhD Symposium (3 day international conference)

- Poster presentation at the 16th EMBL PhD Symposium entitled "Inspired by biology: Exploring natures toolbox" in Heidelberg, Germany
- Poster title: "Anaerobic whole cell microbial biosensors"
- Link: http://phdsymposium.embl.org/#home

September, 2014   Oral presentation to the Department of Microbiology, La Trobe University, Bundoora, Victoria 3086

- Departmental presentation
- Title: "Microbial biosensors for the detection of heavy metals"

July, 2014   Invited speaker at the Australian Society of Microbiology (ASM) annual scientific meeting

- Oral presentation at the Australian Society of Microbiology (ASM) annual scientific meeting in Melbourne, Australia (3 day conference)
- Talk title: "Synthetic biology based microbial biosensors for the detection of heavy metals"
- Link: http://asmicro-2014.p.asnevents.com.au/schedule/abstract/13816

e) manuscripts submitted but not yet published, and
1. Lara Bereza-Malcolm[1], Sanja Aracic[1] and Ashley E. Franks[1]* Investigation of the underlying factors effecting microbial biosensor function using multiple arsenic biosensors
2. Sanja Aracic1, Lara Bereza-Malcolm1, William L. King3, Gülay Mann2, Maurizio Labbate3, and Ashley E. Franks1* Gram-negative Synthetic Biologically derived bacterial biosensors for the detection of mercury

Awards and achievements of PhD student Lara Bereza-Malcolm support inpart by project
1. April, 2016 Synthetic Biology Cutting Edge Symposium
   - Awarded best student poster/oral for the SBA conference
   - Received $200
2. June, 2015 Member of the Winning team of the Australian-French 24-hour Entrepreneur Challenge
   - 24-hour challenge run by the Australian Academy of Science as part of NISA
   - Member of the winning team consisting of 6 Australian PhD students and 1 French PhD student
   - Received a trip to Paris to meet industry and business partners
3. December, 2015 Nominee for 2015/2016 Victorian Young Achiever Award
   - Nominated for the Science & Technology Award and Research Impact Award
4. June-September, 2014   National Three Minute Thesis (3MT) competition
   - Awarded 2nd place and a $2000 travel grant at the La Trobe

University round
- Awarded the people's choice award and a $200 Co-op voucher at the La Trobe University round
- Awarded 2nd place in the second 3MT round (Formally the Faculty of Science, Technology and Engineering round)
- Awarded 2nd place in the first 3MT round (Formally the School of Life Sciences round)

Funding awarded to PhD student Lara Bereza-Malcolm
1. June, 2016 Postgraduate Research Overseas Activity Grant Awarded $500 towards travel to SEED2016: Synthetic Biology, Engineering
2. September, 2015 Sponsorship from Sigma Aldrich Awarded $200 towards a seminar hosted by Supporting Women in Science (SWIS)
3. August, 2015 Graduate Research School Intellectual Climate Grant award Awarded $500 towards the development of the Supporting Women in Science (SWIS) society
4. August, 2014 Awarded a European Molecular Biology Laboratory (EMBL) travel grant Awarded an EMBL Australia travel grant of $3000 to attend the 16th EMBL PhD symposium, titled "Inspired by biology: Exploring natures toolbox," in Heidelberg, Germany

f) provide a list any interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work.
1. Establishment of Synthetic Biology of Australasia (https://synbioaustralasia.org/).
2. A. Franks is the Secretary of the Synthetic Biology of Australasia (https://synbioaustralasia.org/).
3. Presented at Airforce program review in Dayton Ohio – 2015,1016. Development of ongoing collaboration regarding synbio and microbiome.
4. Franks Chair of the awrds committee for International Society for Microbial Technology
5. On going project in conjunction with the Defence Science and Technology Group (Australia)
6. S. Aracic (postdoc) gained ongoing employment in private sector start up company Affinity Bio

Attachments: Publications a), b) and c) listed above if possible.

DD882:   As a separate document, please complete and sign the inventions disclosure form.

Important Note: If the work has been adequately described in refereed publications, submit an abstract as described above and refer the reader to your above List of Publications for details. If a full report needs to be written, then submission of a final report that is very similar to a full length journal article will be sufficient in most cases. This document may be as long or as short as needed to give a fair account of the work performed during the period of performance. There will be variations depending on the scope of the work. As such, there is no length or

formatting constraints for the final report. Keep in mind the amount of funding you received relative to the amount of effort you put into the report. For example, do not submit a $300k report for $50k worth of funding; likewise, do not submit a $50k report for $300k worth of funding. Include as many charts and figures as required to explain the work.